INTRODUCTION

The genome of Sinorhizobium meliloti strain 1021 is predicted to encode 6204 proteins, 59.7% of which have a suggested function (Galibert et al., 2001). This soil bacterium establishes a symbiotic relationship with host plants such as Medicago sativa (alfalfa) and induces the production of specialized organs called nodules. If this association is to be successful, the bacterial population must be maintained in the soil and come into contact with the plants.

Like many other soil bacteria, S. meliloti frequently faces long periods of carbon, nitrogen and phosphorus starvation and is frequently exposed to stresses such as water or O₂ depletion (Thorne & Williams, 1997). Shortly after coming into contact with pathogenic and symbiotic bacteria, plants react by producing an oxidative burst that generates a large amount of ROS (reactive oxygen species), mainly hydrogen peroxide (H₂O₂) (Ramu et al., 2002). H₂O₂ accumulates within 2 or 3 min of contact and declines after 40–50 min in soybeans (Levine et al., 1994).

Abbreviations: CuOOH, cumene hydroperoxide; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; tBOOH, t-butyl hydroperoxide.

Response of S. meliloti to this burst is critical for the control of the infection and the nodulation stages (Santos et al., 2000). Furthermore, during nodule colonization, the bacteria differentiate into bacteroids and produce nitrogenase, which allows them to fix N₂. As the nitrogenase is quickly and irreversibly inactivated by O₂, the oxygen concentration within the cells must be carefully regulated (Dalton et al., 1998). Large quantities of ROS are also formed by aerobic respiration, due to the incomplete reduction of oxygen molecules. ROS damage cellular lipids (e.g. membranes), proteins and nucleic acids, and participate in the degeneration of cells such as the senescence of the bacteroids in the nodule.

The bacterial oxidative stress response involves well-orchestrated reactions, involving two important categories of protein. The first category includes the enzymes and small molecules that directly detoxify (or protect against) peroxides and superoxide ions, for example, superoxide dismutase (SOD) and catalase (Kat). S. meliloti produces three catalases encoded by three genes, each located on a different replicon (Galibert et al., 2001): KatA (smc00819, monofunctional), KatB (sma2379, bifunctional) and KatC (smb20007, monofunctional). Only KatA is induced by

Sequencing of the Sinorhizobium meliloti strain 1021 genome led to the detection of 6204 open reading frames, 41% of which have no hypothetical function. To help annotate this genome, a transcriptome analysis was carried out with a dedicated microarray consisting of 146 genes belonging to three different classes: (i) no hypothetical function; (ii) potentially involved in oxidative stress responses; (iii) known to participate in oxidative stress responses (e.g. catalase and superoxide dismutase genes). This transcriptome analysis, together with biological experiments and in silico investigations, identified new genes induced by exogenous H₂O₂. The smc01944 gene was the most strongly induced: quantitative PCR showed that the amount of smc01944 mRNA increased 50-fold following the addition of 10 mM H₂O₂, whereas the amount of katA mRNA (encoding a catalase) only increased 10-fold. Smc01944 is a non-haem chloroperoxidase (Cpo). The only member of this family to have been so far characterized is encoded by prxC of Pseudomonas fluorescens. Unexpectedly, the NH₂-terminus of Smc01944 includes a signal peptide and Smc01944 is secreted into the supernatant. Interestingly, smc01944 is preceded by smc01945, encoding an OhrR-like regulator (MarR family). Thus, Smc01944 is the first exported Cpo encoded by a gene possibly regulated by an OhrR regulator. It was also shown that smc01944 is induced by t-butyl and cumene hydroperoxides but only slightly by menadione. The study of Smc01944 described in this work showed that the oxidative stress response of S. meliloti seems to differ from that of other bacteria characterized to date.
H₂O₂, whereas KatB is continuously produced and ensures that a low H₂O₂ concentration is maintained during the exponential phase of growth and KatC is induced by osmotic and thermal stresses (Herouart et al., 1996; Sigaud et al., 1999). S. meliloti also expresses two SODs, encoded by the chromosomal genes smc00043 (Soda or SodB; Mn SOD) (Santos et al., 2001) and smc02597 (SodC; Cu/Zn SOD) (Galibert et al., 2001). Interestingly, a third gene, smc00911, encodes a hypothetical protein that is 62 % identical with SodM of Bradyrhizobium japonicum (GenBank accession no. Q9HR60). SODs are essential for effective nodulation (Santos et al., 2001). With the exception of these six genes, no other candidates encoding enzymes like alkylhydroperoxidases or peroxidases have been formally identified in S. meliloti, illustrating the importance of further biological experiments.

The second category of proteins involved in the oxidative stress response includes the regulatory proteins that control the expression of the above-mentioned genes. In Escherichia coli, four key regulatory proteins govern the adaptive response to H₂O₂. The OxyR protein (a 34 kDa LysR-transactivator) (Schellhorn, 1995) and the SoxR–SoxS two-component system (Manchado et al., 2000) are active during the exponential phase of growth, and the sigma factor RpoS is active during the stationary phase. Two candidate genes thought to encode OxyR and SoxR have been identified on the S. meliloti chromosome (Capela et al., 2001). However, neither soxS nor rpoS has been identified in this species.

To identify the regulatory processes that S. meliloti uses when exposed to H₂O₂ stress, we created microarrays containing a subset of 146 genes and studied their transcription levels in cells exposed to 10 mM H₂O₂. This work enabled us (i) to identify three new genes involved in the peroxide stress response, (ii) to confirm the importance of KatA in this response and (iii) to discover new regulatory processes that preserve the redox status of the rhizobial cell.

METHODS

PCR primer design. PCR primers were designed to amplify a 200–400 bp fragment in the 3’ part of 146 of the 6204 S. meliloti genes. These ORFs could be divided into three groups: (i) known to participate in the oxidative stress response (e.g. catalase and SOD genes); (ii) potentially involved in the oxidative stress response; (iii) with no hypothetical function but containing protein domains that could be important for the pathways analysed (e.g. oxidases and dehydrogenases). We designed all primers using Primer 3 software (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi) such that their melting temperature was between 65 and 70 °C. Eurogentec Custom Microarray Service synthesized the primers, carried out the PCRs and spotted target DNA onto CMT-Gaps slides (Corning). All genes were spotted onto each slide in duplicate.

Bacterial growth and total RNA extraction. S. meliloti strain 1021 was used to inoculate 250 ml Vincent minimal medium (Vincent, 1970) in 2 l roller bottles (Greiner). The cells were grown at 30 °C and the OD₆₅₀ measured to determine the growth stage.

H₂O₂ (Sigma) (10 mM final concentration) was added to late-exponential-phase cultures and aliquots were collected after 0, 1, 2, 6, 8 and 10 min and centrifuged at 3000 g. The resulting pellets were frozen until use. Cells were resuspended by vortexing in 200 μl TE (10 mM Tris/HCl pH 8.0, 1 mM EDTA) containing 2 mg lysozyme ml⁻¹ (Sigma). The lysates were treated with RQ1 RNase-free DNase (Promega) at 37 °C for 45 min. Total RNA was then isolated using the Qiagen RNeasy kit, according to the manufacturer’s instructions. For all samples, we carried out two independent RNA extractions.

First-strand cDNA fluorescent labelling. To generate fluorescent cDNA targets, 15 μg of total RNA was used for each labelling reaction. We used 3 μl of random primers (3 μg μl⁻¹, Gibco-BRL) and 3 μl of each primer used to prepare the 146 spotted genes (i.e. 292 primers altogether at 1 nM final concentration each) and 1 mM FluoroLink Cy3-dUTP (Amersham Biosciences) for labelling at 42 °C using the Cyscribe protocol and reagents (Amersham). After labelling, RNA was removed by NaOH/HCl treatment and cDNA was purified with the Qiagen PCR purification kit and eluted in a final volume of 30 μl TE.

Hybridization and washes. Hybridization was carried out in an ArrayIt hybridization cassette (TeleChem International). After 2 min at 100 °C, 17 μl cDNA targets was mixed with 3 μl 20 × SSC and 0.45 μl 10 % SDS and 1 μl sheared salmon sperm DNA (10 μg ml⁻¹) was applied to each slide. The entire hybridization chamber was submerged in a 60 °C water bath for 16 h. The slides were washed successively for 5 min each with 1 × SSC/0.3 % SDS, 0.2 × SSC, and finally with 0.1 × SSC, at room temperature prior to low-speed centrifugation drying.

Data analysis. Microarrays were scanned using a ScanArray 4000 confocal scanner (GSI Lumonics) at a resolution of 5 μm per pixel. Scanning parameters (laser and photomultiplier gains) were adjusted to avoid saturation. Scanned images were saved as 16-bit TIFF files and analysed by quantifying the pixel intensity of each spot using the Scananalyse software (M. Eisen, Stanford). The median signal intensity was determined for each spot and the data sheets were then exported to an Excel table for further processing. The median background intensity was subtracted from the intensity of each fluorescent spot. After log₂ transformation, hierarchical clustering was performed and the results were compared using EPCLUST (see Computer analysis) and Genesight (Biodiscovery). To ensure consistency, we carried out two independent reverse transcription steps per RNA sample. Thus, each data point corresponds to the results of four hybridization experiments (two independent reverse transcriptions using two independent RNA samples).

SYBRGreen-based real-time Quantitative PCR (qPCR). Real-time quantitative RT-PCR (qPCR) was used to validate the data from the microarray experiments. For all the time points, qPCRs were performed using the same RNA preparations as for the microarray experiments. The 25 μl qPCR mixtures contained 10 ng cDNA, 12.5 μl 2 × SYBRgreen master mix (Applied Biosystems) and 300 nM of each gene-specific primer. A melting curve was constructed to verify the quality of the amplicon. Assays were performed in triplicate for each cDNA sample with an Applied Biosystems 7000 instrument. All data were normalized with respect to the glyceraldehyde-3-phosphate dehydrogenase (gap) mRNA using the [ΔΔCt] Delta CT method, User Bulletin 2. As for the microarrays, two independent cDNA samples were prepared for each time point.

Quantitative determination of oxidant detoxification. The detoxification of H₂O₂ was measured by use of semi-quantitative strips (Quantofix Peroxid 25; Macherey-Nagel) and appropriate dilutions. Briefly, a quantofix strip was dropped for 1 s in each
sample, every 5 min for 60 min after the H_2O_2 addition. Then, the color obtained was compared to the reference color scale (supplied by the manufacturer) in order to estimate the remaining H_2O_2 concentration. Aliquots (2 ml) of the same samples were taken and centrifuged at 6000 g for 1 min. Then, 100 μl supernatant was added to 100 μl BM blue POD substrate (Roche). After 15 min, necessary for the blue color development (complete reaction), the peroxidase activity was measured by spectrophotometry at 370 nm.

**Bacterial cell compartmentation.** Supernatants of stressed and control cultures (100 ml each) were collected by centrifugation and precipitated with 11% trichloroacetic acid. The periplasm was recovered by osmotic shock: cells were treated with 3 mM Tris/HCl (pH 8.0)/20% sucrose/1 mM EDTA at room temperature for 10 min. After centrifugation (4000 g), the pellet was resuspended in 1 ml sterile cold water by gently shaking on ice for 10 min. The periplasm fraction was then recovered by centrifugation (4°C, 2000 g). The cytoplasm and insoluble fractions were kept at 4°C for SDS-PAGE analysis.

**Recombinant proteins.** The ORF of the smc01944 gene was PCR-amplified with specific primers: smc01944-upper 5’-gaattcgtgatccAT-GACCAAGTCTCAAGTGAG-3’ and smc01944-flower 5’-gaattcgtgatcGGCCAGCTCTTTGAG-3’, where the lower-case letters correspond to KpnI and BamHI sites. The PCR product was purified, digested and ligated into pQE30 (Qiagen) with an N-terminal six-histidine residue tag. The production of the recombinant protein, which contained a 6His tag at the N-terminus, was induced (in LB medium) in E. coli M15(pREP4) with 1 mM IPTG and purified on a Ni²⁺-NTA column using the Qiagen Ni⁺⁺-NTA resin (Qiagen) according to the manufacturer’s recommendations. The purified protein was then separated on a 12% SDS-polyacrylamide gel (Bio-Rad apparatus), stained with Coomassie blue and the band corresponding to the recombinant protein, which contained a 6His tag at the N-terminus, was excised and used for immunization (Eurogentec). The same procedure was used for the ORF of the smb20964 gene.

**Western blot analyses.** Proteins from each cell fraction were separated by SDS-PAGE in a 10–20% gradient gel (Bio-Rad) and then electroblotted onto a nitrocellulose membrane (Hybond ECL membranes, Amersham). The membrane was blocked with 2% nonfat milk in 1× PBS (phosphate-buffered saline) containing 0.1% Tween 20 and incubated with a 1:10 000 dilution of a rabbit antibody raised against the purified Smc01944 recombinant protein, which contained a 6His tag at the N-terminus, and then washed with 1× PBS containing 0.5% Tween 20, the blot was developed with the ECL Western blotting analysis system (Amersham) and used to expose hyperfilm ECL (Amersham).

**Computer analysis.** DNA and proteins sequences were compared by use of NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast/). Cellular localizations were predicted by PSORT (http://psort.nibb.ac.jp/) and SignalP (http://www.cbs.dtu.dk/services/SignalP-2.0/). Membrane regions were detected by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and TMPred (http://www.ch.embnet.org/software/TMPRED_form.html). Protein motifs were identified by Prodom (http://prodes.toulouse.inra.fr/prodom/doc/formCG.html). The microarray results were clustered by EPCLUST (http://ep.ebi.ac.uk/EP/EPCLUST).

**RESULTS**

**Effect of increasing concentrations of H_2O_2 on S. meliloti 1021 survival and growth**

We investigated the ability of late-exponential-phase cells of *S. meliloti* strain 1021 (OD₆₀₀ 0.8 in MV minimal medium) to respond to exposure to a range of H_2O_2 concentrations (1–75 mM). Survival curves (Fig. 1) showed that more than 50% of the bacteria survived at concentrations of between 1 and 10 mM H_2O_2, probably because *S. meliloti* possesses an efficient detoxification system. At higher concentrations (25–75 mM), the killing rate was higher (>70%) and increased in a dose-dependent manner. Thus, *S. meliloti* cells, grown in MV medium to OD₆₀₀ 0.8, are able to efficiently detoxify 10 mM H_2O_2. Cultures were only irreversibly affected above 25 mM H_2O_2.

**Induction of gene expression by H_2O_2 during the late exponential phase**

The expression levels of the 146 selected genes were monitored after the addition of 10 mM H_2O_2 to a *S. meliloti* 1021 culture (OD₆₀₀ 0.8) (Table 1).

A first group contained six genes that were strongly expressed in the first 10 min after adding H_2O_2. Among these genes, we selected the hypothetical peroxidase smc01944 gene for a biochemical study (this paper) because its expression was induced more strongly than that of the gene encoding the well-characterized catalase KatA and its function is only hypothesized by *in silico* predictions. In fact, katA is the only catalase gene induced by H_2O_2 in *S. meliloti* RM5000 (Herouart et al., 1996). This group also contained smb00400, which encodes a 14 kDa protein with a unique functional domain common to the OsmC-Ohr protein family, and smb20964, predicted to encode a cytoplasmic protein belonging to the AhpC/Thi anti-oxidant protein family as it is 32% identical to *E. coli* alkyl oxidase (Amersham).
hydroperoxide reductase (AhpC). These genes will be the object of further studies.

The second group included 47 genes that showed one or two time-dependent significant expression levels (>3). This group included six genes encoding proteins involved in DNA repair and eight genes encoding glutathione S-transferases. Finally, the third group of 93 genes were only weakly transcribed if at all. In fact, their expression levels did not vary significantly after exposure to H$_2$O$_2$ when compared to group 1. This set included the genes encoding the catalases Smb20007 (corresponding to katB) and Sma2379 (corresponding to katA). The mRNA levels of these genes remained at a constant low level (in agreement with previous results obtained by Herouart et al., 1996) as did that of the gene encoding the putative OxyR (smc00818), a transcriptional regulator of oxidative stress.

Comparison of the induction rates of smc01944 and katA following exposure to oxidants

DNA microarrays are a very useful tool for the detection of genes that are upregulated following exposure to stress, but this technique gives mainly qualitative results. For this reason, we used qPCR (Table 2) to compare the induction levels of smc01944 and katA after exposure to four oxidants: H$_2$O$_2$, vitamin K (menadione), t-butyl hydroperoxide (tBOOH) and cumene hydroperoxide (CuOOH).

Following exposure to H$_2$O$_2$, the induction level of katA remained virtually constant, regardless of dose and time of exposure (approx. 10–13-fold, Table 2). Conversely, the expression level of smc01944 depended on dose and time. In the presence of 5 mM H$_2$O$_2$, the transcription of smc01944 increased 60-fold within 5–10 min. In the presence of 10 mM H$_2$O$_2$, smc01944 expression peaked.
Table 2. Induction level of smc01944 and katA, determined by qPCR experiments

The data represent the ratio between unstressed and stressed cells of S. meliloti. Ratios above 3 are in bold.

<table>
<thead>
<tr>
<th>Oxidative stress</th>
<th>qPCR ratio</th>
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<tbody>
<tr>
<td></td>
<td>smc01944</td>
<td>katA</td>
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<tr>
<td>H2O2</td>
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</tr>
<tr>
<td>5 mM, 5 min</td>
<td>12·7</td>
<td>12·9</td>
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<tr>
<td>5 mM, 10 min</td>
<td>60</td>
<td>12·8</td>
</tr>
<tr>
<td>10 mM, 5 min</td>
<td>70</td>
<td>12·8</td>
</tr>
<tr>
<td>10 mM, 10 min</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>tBOOH</td>
<td></td>
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<tr>
<td>1 mM, 5 min</td>
<td>9·5</td>
<td>2·3</td>
</tr>
<tr>
<td>1 mM, 10 min</td>
<td>43·4</td>
<td>3·6</td>
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<tr>
<td>5 mM, 5 min</td>
<td>11</td>
<td>2·4</td>
</tr>
<tr>
<td>5 mM, 10 min</td>
<td>37·3</td>
<td>3·4</td>
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<tr>
<td>CuOOH</td>
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<tr>
<td>1 mM, 5 min</td>
<td>9·5</td>
<td>2·5</td>
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<tr>
<td>1 mM, 10 min</td>
<td>43</td>
<td>1·8</td>
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<tr>
<td>5 mM, 5 min</td>
<td>7·6</td>
<td>2·5</td>
</tr>
<tr>
<td>5 mM, 10 min</td>
<td>28·8</td>
<td>2·9</td>
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<tr>
<td>Menadione</td>
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<tr>
<td>1 mM, 5 min</td>
<td>12·8</td>
<td>2·4</td>
</tr>
<tr>
<td>1 mM, 10 min</td>
<td>1·6</td>
<td>2·6</td>
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after 5 min (70-fold increase) but then decreased at 10 min. This indicates that the regulation of smc01944 depends on the amount of peroxide present, whereas that of katA does not.

The level of katA mRNA remained constantly low in the presence of the organic hydroperoxides (tBOOH and CuOOH), which showed that katA seems to be induced in a similar manner to other bacterial catalase genes following exposure to H2O2. In contrast, the transcription of smc01944 responded to both tBOOH and CuOOH. The induction profiles of smc01944 were very similar for both tBOOH and CuOOH. Expression levels increased with time (by about 40-fold after 10 min). These stimulations resemble, in a lesser way, that observed for H2O2.

Finally, a superoxide generator (1 mM menadione) induced smc01944 expression by about 13-fold at 5 min but not after 10 min, suggesting that in our experiment, menadione acts by another unknown way than as superoxide generator.

Characterization of Smc01944

Sequence homology searches revealed that this protein contains a non-haem haloperoxidase domain signature (EC 1.11.1.–) and is 74% identical with the chloroperoxidase PrxC, encoded by the cpoF gene of Pseudomonas fluorescens (Kirner et al., 1996).

Smc01944 has at least two paralogues in the whole genome of S. meliloti: the proteins encoded by smb20054 and smb20860 are 64% and 68% identical with PrxC, respectively. The expression of these genes was only increased three- to fourfold by 10 mM H2O2 (Table 1). The expression levels of the two other genes potentially encoding S. meliloti peroxidases (sma2031 and sma1809) were not significantly increased by 10 mM H2O2 (Table 1).

BLASTP searches of the 110 sequenced bacterial genomes (available in the whole genome NCBI-BLAST database) revealed the presence of Smc01944 orthologues in environmental bacteria like Mezorhizobium, Burkholderia fungorum, Xanthomonas spp., Pseudomonas spp., Agrobacterium and Bacillus subtilis but not in E. coli K-12 or O157. Based on its gene sequence, the N-terminal part of Smc01944 would contain 57 extra amino acids compared to the seven other chloroperoxidases from the same branch (Fig. 2). To eliminate the possibility of sequencing errors, we resequenced this gene using genomic DNA from strain 1021 and the related strains 2011 and SU47 (data not shown) and confirmed the existence of this additional sequence. Both the SignalP and TMHMM algorithms found that this sequence is a 44 amino acid signal peptide with a transmembrane segment (positions 20–42) and a cleavage site at positions 42–43 (AVA-GG). This signal peptide

Fig. 2. Multiple alignment of the N-terminal region of CpoF-like proteins from S. meliloti strain 1021 (Smc01944 and Smb20054) with other related non-haem chloroperoxidases from Agrobacterium tumefaciens (NP_357147 and NP_357116 strain C58-Cereon; AAL45757 strain C58-U.Washington), Mezorhizobium loti (NP_102663), Xanthomonas campestris strain ATCC 33913 (NP_637528) and CpoF of Pseudomonas fluorescens (AAB86577). The 57 extra amino acids that form the signal peptide are clearly visible. The putative tat-recognition sites are underlined and arrowheads indicate the putative signal peptidase sites.
has a TAT-recognition motif (SRREIL) immediately in front of the transmembrane fragment. The presence of this motif associated with a 44 amino acid peptide signal suggests that Smc01944 is addressed to the periplasm by a SEC-independent TAT system before possibly being secreted into the external medium by the type II general secretion pathway (Sandkvist, 2001).

To determine whether Smc01944 is indeed found in the periplasm and then secreted into the culture supernatant, we carried out Western blotting with proteins extracted from each of the three components (pellet, periplasm and supernatant). A polyclonal antibody raised against recombinant Smc01944 specifically detected one protein of approximately 36 kDa in the pellet, periplasm and supernatant of a stressed culture (Fig. 3a). A control showed that the cytoplasmic protein Smb20964 was not significantly present in the supernatant or periplasm, indicating that this result was not an artefact due to bacterial lysis (Fig. 3b). This strongly suggests that Smc01944 is specifically involved in the detoxification of external H₂O₂.

To test the peroxidase activity of the secreted form of Smc01944, supernatants were collected 0, 1, 5, 10, 30 and 60 min after the addition of H₂O₂. The amount of H₂O₂ remaining in the collected fraction was measured and adjusted to a final concentration of 10 mM. The peroxidase activity of each supernatant was followed by spectrophotometry using BM blue POD as a substrate (Fig. 4a). This experiment detected peroxidase activity in the supernatant of S. meliloti and showed that this was triggered by H₂O₂ stress. The enzymic activity increased rapidly (peaking 5 min after the addition of H₂O₂) and remained almost stable until 30 min. Residual peroxidase activity could still be observed after 60 min. As this pattern was well correlated with the production and secretion kinetics of Smc01944, as shown by the immunodetection experiments (Fig. 4b), and as no other peroxidase genes were significantly induced by H₂O₂ stress (microarray data, Table 1), Smc01944 must display peroxidase activity.

We also compared the amount of Smc01944 produced following exposure to organic peroxides, as qPCR indicated that the transcription of the corresponding gene increased significantly after exposure to CuOOH, tBOOH and menadione. The results (Fig. 5) revealed a correlation between the oxidant used, smc01944 transcription and the production of Smc01944; this protein could not be detected after paraquat stress, an agent that did not increase the amount of smc01944 mRNA (preliminary data). Immunodetection experiments also revealed a big difference between the quantity of Smc01944 produced after exposure to organic hydroperoxides (CuOOH, tBOOH) and after exposure to H₂O₂ (at least 10- to 20-fold less Smc01944 was produced in H₂O₂-treated samples than in organic peroxide-treated samples). Furthermore, in this test, we loaded three times less material than for the induction course (Fig. 3), which explains the intensity discrepancies between the two immunodetection analyses.

An additional band at 49·4 kDa was observed in every sample exposed to CuOOH (weak band) and 1 mM tBOOH (strong band). It is difficult to speculate about the nature of this band, as Smc01944 is the only chloroperoxidase known to be secreted. It is possible that, in certain stress conditions, this protein is extruded together with its...
cofactor, which is consistent with the gel mobility shift observed. This phenomenon will be the object of a future study.

Finally, we should note the presence of smc01945, located downstream of smc01944 and in the opposite direction, encoding a protein that exhibits significant similarities with the OhrR regulators (MarR family) of Bacillus subtilis and Xanthomonas campestris (46% and 55% identity, respectively), especially around the cysteine residue present in the N-terminal part (QLCF motif). This is the cysteine residue that, when oxidized, changes the affinity of OhrR for its DNA-binding site, alleviating transcriptional repression (Mongkolsuk & Helmann, 2002). The importance of smc01945 in the transcriptional regulation of smc01944 will be studied further.

DISCUSSION

The production of oxidizing compounds (ROS) is one of the early mechanisms of eukaryotic (plants and animals) defence strategies against pathogenic prokaryotes. During the early events of symbiosis, the plant hosts, in particular alfalfa, produce ROS (H$_2$O$_2$, superoxide radicals) in response to contact with S. meliloti (Santos et al., 2001). In E. coli, two-dimensional gel electrophoresis has shown that approximately 30 major proteins are induced by H$_2$O$_2$; 12 of these are induced in the first 10 min and the 18 others are induced between 10 and 30 min (Zheng et al., 2001). In S. meliloti, only six genes were dramatically induced in our dedicated microarray but preliminary experiments using S. meliloti pangenomic microarrays (work in progress) have indicated that as many as 100 genes are concerned.

Our work suggests that one of the most important responses of S. meliloti to H$_2$O$_2$, the main ROS encountered during rhizospheric and symbiotic life, is the expression of a gene encoding a chloroperoxidase (smc01944). This gene is expressed fivefold more strongly than the gene encoding the KatA catalase, which was considered as the main peroxidase in this bacterium. The second characteristic of this response is the secretion of Smc01944 into the periplasm and the external medium, enabling this enzyme to detoxify all three compartments simultaneously. Recent studies showed that hydroperoxides can be dismutated by the catalase-like activity of Smc01944 with the following conversion rates: H$_2$O$_2$ > tBOOH > CuOOH (Manoj & Hager, 2001). This analysis showed that in comparable conditions, Smc01944 converted 95% of H$_2$O$_2$ in 30 s, 15% of tBOOH in 10 min and only 1% of CuOOH in 10 min. A similar preference order was found in our qPCR quantification for the transcription induction of smc01944. Thus, we hypothesize that the strong affinity of Smc01944 for H$_2$O$_2$ involves its fast and transitory induction and explains the weak accumulation of the protein during this peroxide stress. In comparison, as organic hydroperoxides are less rapidly degraded, smc01944 is induced for a longer period of time and Smc01944 accumulates in the cytoplasm, periplasm and external medium as shown by our Western blots.

Thus, Smc01944 may be an important detoxification system, partly due to the fact that this protein is able to detoxify the cytoplasm, the periplasm and the external medium. Given that the doubling time of S. meliloti is approximately 3 h, it is possible that its antioxidant machinery prioritizes the decontamination of external oxidative stress rather than internal stress, which is mainly generated by respiratory processes. The opposite is probably true in organisms that have a faster doubling time, like E. coli.

The weak activation of katA observed in our experiments may have been due to the high level of activity of Smc01944 in the periplasm and in the external medium, which would have prevented the oxidant from entering the cytoplasm and to some extent activating oxyR. With this

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**Fig. 5.** Immunodetection of Smc01944 in S. meliloti pellets after exposure to different concentrations of tBOOH, CuOOH, paraquat, menadione and H$_2$O$_2$. Three times less pellet material was loaded than in Fig. 4 (50 ml treated culture instead of 150 ml).
modus operandi, external H$_2$O$_2$ would be broken down by Smc01944 more rapidly than it can diffuse through the cytoplasmic membrane.

Our study shows that the major importance of KatA in the response to the exogenic peroxide stress is attenuated by the production and the secretion of Smc01944, which could explain why _S. meliloti_ katA mutants (i) are still able to produce the same number of healthy nodules as the wild-type, (ii) can retain a Fix$^+$ phenotype and (iii) do not induce the katB and katC genes notably (Herouart _et al._, 1996; Sigaud _et al._, 1999).

Our analysis suggests a particular mode of regulation for _katA_ and _smc01944_, in which one regulatory protein controls each enzyme gene rather than a large regulon like the _E. coli_ OxyR regulon (Zheng _et al._, 2001). This mode of control probably increases the possibility to adapt to the various ecological niches occupied by this bacterium (e.g. soil, rhizosphere, nodules).

**ACKNOWLEDGEMENTS**

We would like to thank Marie-Dominique Galibert and Nadia Berkova for their technical assistance and fruitful advice. The CNRS DNA microarray programme supported this work.

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